Effects of Dietary Nitrogen Source on Concentrations of Ammonia, Free Amino Acids and Fluorescaminereactive Peptides in the Sheep Rumen

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EFFECTS OF DIETARY NITROGEN SOURCE ON CONCENTRATIONS OF AMMONIA, FREE AMINO ACIDS AND FLUORESCAMINE-REACTIVE PEPTIDES IN THE SHEEP RUMEN[1,2]

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ABSTRACT

Four sheep were fed twice daily a diet of 67% ryegrass hay and 33% concentrate during three, 4-wk periods. The diet was supplemented with one of three N sources: urea, casein or ovalbumin. Urea was the only supplemental N source during Period 1. Sheep were fed either casein or ovalbumin during Period 2, followed by switching of the supplements during Period 3. Statistical comparisons were made only on data obtained during Periods 2 and 3. Ruminal concentrations of NH_3, free amino acids and peptides were measured over an 8-h period after feeding. The peptide assay was based on the enhanced fluorescence of peptides, relative to amino acids, obtained from reaction with fluorescamine at pH 6.2. Ammonia accumulated rapidly to high concentrations (maximum 38 m*M* at 1 h) after feeding urea. Ammonia was intermediate with casein and greater ($P < .05$) than with ovalbumin. Free amino acids were greater ($P < .05$) with casein (maximum 1.4 m*M* at 1 h) than with ovalbumin. Free amino acids were intermediate with feeding of urea. Only transient accumulation of peptides occurred when casein was fed: ruminal peptides increased to 3.8 m*M* at 1 h, but declined to prefeeding levels (.2 m*M*) by 3 h. Peptides in ruminal fluid from sheep fed ovalbumin were lower ($P < .01$) and (similar to urea) did not change from prefeeding levels (.2 to .3 m*M*) throughout the sampling period. It was concluded that slowly degraded proteins, such as ovalbumin, will not give rise to significant peptide levels, whereas rapidly degraded proteins, such as casein, will yield substantial levels of peptides during ruminal protein degradation.

(Key Words: Urea, Casein, Egg Albumin, Proteins, Rumen Metabolism, Ammonia, Free Amino Acids, Peptides, Fluorescence.)

Introduction

Ammonia is the main precursor for microbial protein synthesis in the rumen (Nolan, 1975) and, although the required concentration remains controversial (Satter and Slyter, 1974), NH_3 must be present in excess of microbial requirements for optimal fermentation to occur (Odle and Schaefer, 1987). Free amino acids (FAA) are formed during protein degradation (Broderick, 1978) and may stimulate microbial protein synthesis (Maeng et al., 1976). Inhibition of ruminal amino acid deamination by feeding ionophores or other compounds has been suggested as a way of improving N utilization in ruminants (Chalupa, 1980).

The most heterogenous, and least understood, intermediates of ruminal protein catabolism are peptides. Peptides are required for growth of *Bacteroides ruminicola* (Pittman and Bryant,

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1964) and with mixed ruminal organisms result in more rapid growth than from NH_3 alone (Wright, 1967). Mixed ruminal organisms degrade peptide-bound amino acids more rapidly than FAA (Prins, 1977; Prins et al., 1979). Transient accumulation of peptides occurred during the breakdown of protein in vitro (Broderick and Craig, 1983; Russell et al., 1983), but peptides did not accumulate during degradation of leaf fraction 1 protein in vivo (Nugent and Mangan, 1981).

This paper describes development of a method for fluorimetric determination of peptides in ruminal fluid and application of this method to study how different sources of dietary N influence accumulation of peptides as well as FAA and NH_3 in the rumen.

Materials and Methods

This experiment was part of a study on the effects of feeding specific proteins on the degradative activity of ruminal microbes (Wallace et al., 1987). Four sheep, weighing 50 to 55 kg and fitted with permanent ruminal cannulas, were fed twice daily (at 0800 and 1600) .5 kg (1.0 kg/d) of air-dry diet consisting of 67% ryegrass hay and 33% concentrate. The concentrate mix (Whitelaw et al., 1983) contained 53% corn, 14% oats, 14% wheat bran, 1% vitamin and mineral concentrate, and either 5.4% urea (plus 12.6% corn), 18% casein[5] or 18% ovalbumin[5]. Total CP of these diets was calculated to be 15.6% (DM basis). Urea, casein and ovalbumin provided 32, 34 and 34% of the total CP in the respective diets. The trial consisted of three, 4-wk feeding periods. All sheep were fed the urea diet for the first (preliminary) period. Then, two sheep were switched to the casein diet and two to the ovalbumin diet for the second period. Finally, the casein and ovalbumin diets were switched during the third (last) period of the trial. Ruminal contents were sampled on the penultimate day of each period via cannulas just prior to feeding (at 0800), and at 1, 2, 3, 4, 6 and 8 h after feeding; contents were filtered through four layers of muslin and held on ice. Samples were processed within 10 min of collection by mixing 8 ml of strained ruminal fluid (SRF) with two ml 25% w/v trichloroacetic acid, holding on ice for 30 min, and centrifuging (27,200 × *g*, 4°C, 30 min). Supernatant fluids were stored at −20°C until analyzed.

Samples were thawed and recentrifuged (27,200 × *g*, 4°C, 15 min). Supernatant fluids were assayed in duplicate for NH_3 by a phenol-hypochlorite method (Weatherburn, 1967) and for total FAA (alpha-amino N) with an automated, trinitrobenzene sulfonic acid procedure that does not respond either to urea or NH_3 (Palmer and Peters, 1969).

Peptide concentrations were determined in SRF supernatants by methods similar to those of Perrett et al. (1975) and Nisbet and Payne (1979). Response of individual amino acids in this procedure was typically only 1 to 2% that of corresponding peptides. Glycine, the free amino acid showing the greatest response, yielded 8 and 5% of the fluorescence of di- and triglycine, respectively. Supernatant fluid (75 μl) was added to 2.25 ml .20 *M* sodium citrate buffer (pH 6.2). Just prior to fluorescence analysis, .75 ml of freshly prepared fluorescamine[5] solution (.28 mg/ml in acetone) was added while sample and citrate buffer were mixed by vortexing. After mixing for an additional 3 to 4 s, fluorescence was measured using a Baird Nova fluorometer[6], with excitation at 390 nm and emission at 485 nm. Response was stable between 2 to 20 min after fluorescamine addition. Although fluorescence differed for specific peptides[5] (Table 1), response was linear for each peptide studied. Trialanine response was typical for the tri- and oligopeptides tested; therefore, it was selected as standard and SRF peptides are reported as trialanine equivalents. Mean recovery of trialanine added to SRF was 93.4 ± 3.0 (SE)%; peptide concentrations in SRF were corrected based on this recovery. Standard solutions of quinine sulfate[5] in .1 *N* H_2SO_4 were run with each set of standards and samples.

The first period, when sheep were fed supplemental N only from urea, was considered a preliminary period and was not used in statistical analysis. Data from this period are included in the figures for comparative purposes. Ruminal metabolite concentrations from the cross-over portion of the experiment (Periods 2 and 3) were subjected to a split-plot analysis (Steel and Torrie, 1960) using the GLM procedure of SAS (1985). The main plot was

[5] Sigma Chemical Ltd., Poole, Dorset BH17 7NH, England.

[6] Baird-Atomic Ltd., Braintree, Essex CM7 7YL, England.

TABLE 1. RELATIVE FLUORESCENCE OF SPECIFIC PEPTIDES IN FLUORESCAMINE ASSAY

Peptide[a]	Relative response[b] $\bar{X}$	SE
Dipeptides		
GlyAla	6.7	.3
GlyGly	7.7	.2
GlyPro	8.1	.3
AlaAla	9.4	.2
ValAla	17.7	.7
Tri- and Oligopeptides		
AlaGlyGly	3.7	.5
GlyGlyAla	8.2	.9
GlyGlyLeu	9.2	.4
GlyAlaAla	10.0	.1
GlyGlyPhe	11.2	.9
AlaAlaAla	12.1	.4
AlaAlaAlaAla	12.1	.2
AlaAlaAlaAlaAla	12.4	.3
LeuGlyGly	14.3	.6
PheGlyGly	27.1	1.5

[a]All optically active amino acid residues in peptides were of the L-configuration.

[b]Fluorimetric response (FR) of peptide relative to that of quinine sulfate. Relative response = [(FR/μmol peptide)/(FR/μg quinine sulfate)].

analyzed for treatment (protein), period and treatment × period interaction effects. The subplot was tested for time, time × treatment, time × period and time × treatment × period effects. The change in metabolite concentrations from 1 to 8 h after feeding each protein was analyzed for linear, quadratic and cubic effects.

Results and Discussion

The colorimetric assays for NH_3 and FAA employed in our study are well established. However, determination of low concentrations of peptides in the presence of amino acids has proven difficult. Although fluorescamine is sensitive to both amino acids and peptides, the reagent reacts only with unprotonated amino groups (Udenfriend et al., 1971). The fluorescamine peptide method takes advantage of greater fluorescamine reactivity of peptides than amino acids resulting from lower pKa and hence lower protonation of peptides at acid pH (Perrett et al., 1975). The relative fluorescence (with quinine sulfate as standard) of most of the peptides tested fell in the range 7 to 12 (Table 1). Response to trialanine was typical of the tri- and oligopeptide, so it was used as the standard. Fluorescence of tetra- and pentaalanine was the same as that of trialanine, suggesting that response would not change for peptides when length exceeds three amino acid residues. It is not known why the response of alanyldiglycine was lower; however, higher fluorescence of leucyldiglycine, valylalanine and phenylalanyldiglycine may reflect greater reactivity of peptides with aliphatic or aromatic (i.e., more non-polar) N-terminal amino acids. Mean (SE) peptide concentration in Bactocasitone[7], a source of mixed peptides made from partial enzymatic digestion of casein, was determined to be 1.65 (.08) μmol trialanine equivalents/mg DM. Bactocasitone used in this study contained 12.1% N (DM basis), which corresponds to 13.6 μmol peptides/mg N. The total amino acid content of casein is 54.2 μmol/mg N (Block and Weiss, 1956). The average peptide size may be computed:

$$(54.2\ \mu\text{mol/mg N})/(13.6\ \mu\text{mol/mg N}) =$$
$$4.0\ \text{amino acids/peptide.}$$

By 1 h after feeding urea, ruminal NH_3 was very high (Figure 1); NH_3 concentration declined rapidly thereafter in a first-order manner ($r = -.990$, fractional rate = .200 h^{-1}). Ammonia levels were similar between urea-fed and ovalbumin-fed sheep by 4 h (Figure 1). Overall, NH_3 was greater ($P < .05$) when sheep were fed casein than when fed ovalbumin during Periods 2 and 3. As expected, there were effects ($P < .001$) due to time after feeding and a time × protein interaction ($P < .001$). Ammonia was maximal at 2 h and 3 to 4 h with casein and ovalbumin, respectively. The change in NH_3 from 1 to 8 h after feeding casein had linear, quadratic and cubic effects ($P < .01$), whereas the change in NH_3 after feeding ovalbumin was linear ($P < .05$) over the same time period. Concentrations on all three diets had returned to prefeeding levels by 6 h. The more rapid accumulation of NH_3 with casein than with ovalbumin reflects the known greater resistance of ovalbumin to ruminal degradation (Mangan, 1972; Wallace et al., 1987).

[7] Difco Laboratories, Detroit, MI.

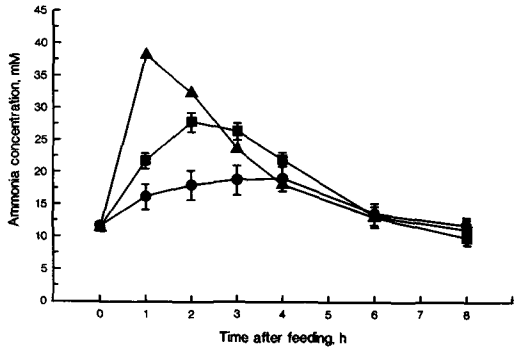

Figure 1. Change in concentrations of NH_3 in strained ruminal fluid after sheep were fed urea (▲), casein (■) or ovalbumin (●). Vertical bars on casein (■) and ovalbumin (●) values represent ± 1 SE.

Concentrations of FAA (Figure 2) were much lower than NH_3; the highest level (with casein) was 1.4 m*M* vs maximal NH_3 (with urea) of 38 m*M*. Relative concentrations of NH_3 and FAA similar to ours were found in extracellular ruminal fluid by other workers (Lewis, 1962; Wallace, 1979; Rodriguez et al., 1986). Concentrations of FAA were greater ($P < .05$) with dietary casein than with ovalbumin; there was an effect due to time after feeding ($P < .001$), as well as time × period ($P < .005$) and time × protein ($P < .001$) interactions during Periods 2 and 3. Free amino acids peaked rapidly in sheep fed casein, before declining rapidly; the FAA pattern with casein had linear and quadratic effects ($P < .001$). It is surprising that FAA levels were greater after feeding urea than after feeding ovalbumin. Broderick et al. (1981) also observed increased FAA concentrations in SRF when urea was fed to increase ruminal NH_3. Ovalbumin feeding did not change FAA from prefeeding concentrations. Broderick and Craig (1983) found similar relative levels of FAA during in vitro incubations of casein and bovine serum albumin, a protein which, like ovalbumin, is soluble but slowly degraded.

Ruminal concentrations of peptides, determined using the fluorescamine method, were greater ($P < .01$) when sheep were fed casein than when fed ovalbumin (Figure 3). Again, there was an effect ($P < .001$) due to time and a time × protein interaction ($P < .001$) during Periods 2 and 3. Peptides were low (about .2 m*M*) and essentially constant on the urea and ovalbumin diets. However, peptides increased dramatically to 3.8 m*M* at 1 h with casein feeding before declining to 1.24 m*M* at 2 h. The pattern of peptide concentrations from 1 h to 8 h after feeding casein had linear, quadratic and cubic effects ($P < .001$). By 3 h, peptide concentrations with casein feeding had returned to prefeeding levels and were similar on all three diets. Others have observed large increases in peptides from ruminal casein breakdown in vitro (Broderick and Craig, 1983; Russell et al., 1983) and in vivo (Chen et al., 1987); peptides did not accumulate during in vitro bovine serum albumin degradation (Broderick and Craig, 1983).

The rapid in vivo clearance of casein peptides in our study is interesting. Russell et al. (1983) reported that peptides from in vitro casein degradation remained elevated after 7 h of incubation. However, they used dilute micro-

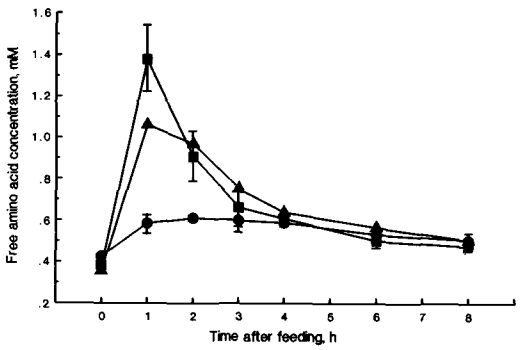

Figure 2. Change in concentrations of total free amino acids in strained ruminal fluid after sheep were fed urea (▲), casein (■) or ovalbumin (●). Vertical bars on casein (■) and ovalbumin (●) values represent ± 1 SE.

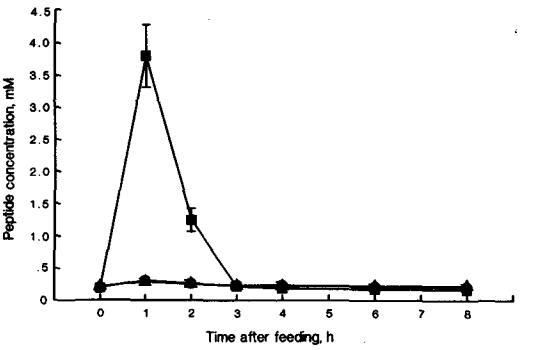

Figure 3. Change in concentrations of peptides (determined by the fluorescamine assay) in strained ruminal fluid after sheep were fed urea (▲), casein (■) or ovalbumin (●). Vertical bars on casein (■) and ovalbumin (●) values represent ± 1 SE.

bial concentrations in their incubations. When expressed per unit of microbial DM, rates of uptake of mixed casein peptides found by Russell et al. (1983) corresponded to about 110 μg N/(d • mg DM). Uptake of alanine peptides observed by Broderick et al. (1987) were equivalent to 73 to 95 μg N/(d • mg DM). Chen et al. (1987) also reported ruminal accumulation of peptides in vivo with soybean meal feeding. Nugent and Mangan (1981) found that peptides did not accumulate during ruminal degradation of fraction 1 protein. However, Hazelwood et al. (1981) observed peptide formation and subsequent hydrolysis to FAA when *Bacteroides ruminicola* was incubated with fraction 1 protein.

We estimated the contribution of FAA and peptides to ruminal non-NH_3-N (NAN) outflow. It was assumed that similar concentration patterns occurred over both feeding cycles, that 6-h levels occurred for 2 h of each feeding cycle, and that 8-h levels applied for all times between 6 h and 0 h (just before feeding). Mean concentrations (m*M*) of FAA and peptides were computed to be .63 and .23 (urea), .61 and .57 (casein) and .54 and .23 (ovalbumin). Based on the liquid volume and passage of 5.3 liters and .08/h (Owens and Goetsch, 1988), liquid passage rate was assumed to be 10 liters/d. Flows (mmol/d) of FAA and peptides were converted to N flows (g/d) assuming the amino acid/N ratio of casein, 54.2 mmol/g N (Block and Weiss, 1956), and four amino acids/peptide. Flow of NAN was assumed to equal N intake (22.5 g/d). Proportion (%) of NAN leaving the rumen as FAA and peptides was estimated to be .5 and .8 (urea), .5 and 1.9 (casein) and .4 and .7 (ovalbumin). These calculations suggest that 1.2 to 2.4% of ruminal NAN flow was contributed by FAA and peptides. Chen et al. (1987) studied the influence of source and amount of dietary protein on ruminal peptide passage in lactating dairy cows. They reported that ruminal peptide passage ranged from 14 to 34 g N/d, with greatest flows occurring with greatest intakes of degradable protein. If NAN flow is assumed to equal N intake, their data yield estimates of 2.7 to 6.7% (avg 4.7%) of NAN leaving the rumen as peptide N. Therefore, the peptide flows of Chen et al. (1987) correspond to values about two to three times greater than those computed from our data.

The present experiment demonstrated that the accumulation of intermediates in the conversion of protein to NH_3 depends on the nature of the dietary protein. Casein is more rapidly degraded than most other proteins, whereas albumins are among the most slowly degraded soluble proteins (Wallace, 1983). Ovalbumin was reported to be broken down at 6% of the rate of casein (Wallace et al., 1987). It seems likely that for most proteins with intermediate rates of degradation, there would be only small, transient accumulation of peptides and FAA immediately after feeding.

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